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14. ABSTRACT
IAP (Inhibitor of Apoptosis Protein) suppresses apoptosis through binding and inhibiting active caspases-3, -7 and -9 via its BIR (baculoviral IAP repeat) domains. During apoptosis the caspase inhibition by IAPs can be negatively regulated by a mitochondrial protein Smac. Smac physically interacts with multiple IAPs and relieves their inhibitory effect on caspases-3, -7 and -9. Recently, a small molecule Smac-mimic compound (Smac-mimic), which potentiates TRAIL and TNF-a mediated cell death in glioblastoma T98G cells and HeLa cells, was identified and characterized. To determine the efficacy of this compound in breast cancer cells, we first measured protein expression of three IAPs: XIAP, cIAP-1, and cIAP -2 in 9 independent breast cancer cell lines. Three cell lines were chosen: a high IAPs expressing line MDA-MB-231, and two low IAPs expressing lines, T47D and MDA-MB-453. The cell lines were tested for their sensitivity to Smac-mimic alone or in combination with TRAIL or etoposide. Acting alone, Smac-mimic was quite potent with a cytotoxic IC50 of 3.8 nM in high IAPs expressing MDA-MB-231 cells, but was inactive at a much higher concentration in low IAPs expressing T47D and MDA-MB-453 cells. In fact, as low as 2.5 nM of Smac-mimic alone was sufficient to activate caspase-3 and induce apoptosis in MDA-MB-231 cells. In combinational treatments with TRAIL or etoposide, Smac-mimic significantly sensitized cells to growth suppression in MDA-MB-231 cells, but to a lesser extent, in T47D and MDA-MB-453 cells. Furthermore, it significantly synergized MDA-MB-231, but not T47D cells to apoptosis induced by either TRAIL or etoposide. Thus, in these cell lines, Smac-mimic acts in an apparent IAPs dependent manner to induce apoptosis alone as well as sensitizes breast cancer cells to TRAIL or etoposide induced apoptosis via caspase 3 activation.

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Introduction

IAP (Inhibitor of Apoptosis Protein), with at least eight family members, suppresses apoptosis through binding and inhibiting active caspases 3/7 and 9 via its BIR (baculovirual IAP repeat) domains. Over-expression of IAPs, particularly survivin is seen in most common cancers (including breast cancers), whereas down-reguation of XIAP or survivin induces apoptosis in multiple human cancer cell lines. Moreover, high levels of survivin are associated with worse clinical outcome (1). In apoptotic cells, the caspase inhibition by IAPs is negatively regulated by a mitochondrial protein Smac. Smac physically interacts with multiple IAPs and relieves their inhibitory effect on caspases 3/7 and 9 (2). The crystal structure showed that this is mainly achieved through the N-terminal four conserved residues (AVPI) in mature Smac molecule that recognize and bind to a surface groove on BIR3 of XIAP (3). Indeed, synthetic Smac peptides with AVPI in its N-terminus is sufficient to activate procaspase-3 (2, 3) and sensitizes cancer cells to apoptosis induced by chemotherapeutic agents in breast cancer cells (4). Since the molecular weight of tetrapeptide AVPI is quite small and structure of their binding to XIAP is available, it is feasible to set up a binding assay to screen for small molecules that compete with these tetrapeptides for XIAP binding, thus activating caspases and inducing apoptosis. The objective of this award proposal is to identify selective, small molecular compounds that mimic Smac's binding to IAP and eliminate IAP's inhibition on active caspases, thus inducing apoptosis in breast cancers and identified small molecule will be characterized for caspase activation, apoptosis induction and/or radio-/chemo-sensitization in breast cancer cells.

Just prior to conducting proposed study, a paper published in Science reporting such a small molecule Smac-mimic compound that is active in disrupting IAP-caspases binding and in sensitizing TRAIL- and TNF-α- mediated cell death in glioblastoma T98G cells and HeLa cells (5). To avoid duplicate work, we decided to characterize this small molecule in its activity against breast cancer cells. As shown in below, this Smac-mimic compound is very potent in inducing apoptosis in the tested breast cancer cells alone or in combination with TRAIL or etoposide in a manner dependent upon cellular levels of IAPs.

Body

Expression of IAPs varies among breast cancer cell lines:

A newly identified Smac-mimic small molecule compound (Smac-mimic) acts by targeting IAPs to relieve their suppression of caspase activity (5). This mechanism of action suggests that Smac-mimic could have a higher efficacy in inducing apoptosis in cancer cells with over-expressed IAPs. To determine efficacy of Smac-mimic in breast cancer cells and to establish a potential correlation between levels of IAPs and drug sensitivity, a total of 9 breast cancer cell lines were examined by western blotting analysis for their protein levels of three well-studied IAPs: XIAP, cIAP-1, and cIAP-2. Expression of IAPs varies significantly among these lines. Three cell lines were selected to determine their sensitivity to Smac-mimic: MDA-MB-231, a high IAPs expresser, MDA-MB-453 and T47D, two low IAPs expressers.

The levels of endogenous IAPs determine cellular sensitivity to Smac-mimic alone or in combination with TRAIL or etoposide:

We next determined, using a 5-day cell proliferation MTS assay, the sensitivity of these three cell lines to Smac-mimic, TRAIL (a ligand for TRAIL receptors to initiate the death receptor apoptosis pathway), or etoposide (a DNA damaging agent to activate the mitochondrial-initiated apoptosis pathway) alone and in combination. The drug concentration used was pre-determined so that it did not induce significant growth suppression when used alone, thus a synergic effect of the drug combinations can be seen. In high IAPs expressing MDA-MB-231 cells, TRAIL and etoposide alone at a concentration of 50 ng/ml and 500 nM, respectively, induced 10-15% growth inhibition (85-90% cell

viability, compared to DMSO control), whereas Smac-mimic caused 40% inhibition at a concentration of 10 nM. When used in combination, Smac-mimic and TRAIL caused up to 100% growth suppression, whereas Smac-mimic and etoposide inhibited growth up to 80%. These results indicated that not only was there a synergic effect of Smac-mimic on agents that activate either death receptor or mitochondrial apoptosis pathways, but Smac-mimic alone had some effect.

In contrast, both low IAPs expressing cell lines, T47D and MDA-MB-453 were quite resistant to these drugs when given alone, although in T47D cells etoposide did inihibit cell growth by 40%. In the case of Smac-mimic, 5-fold higher drug concentration (50 nM) did not induce any growth inhibition in either low IAPs expressing cell line. Combinations of Smac-mimic at this high concentration with TRAIL or etoposide gave rise to a moderate synergic effect in T47D. A relatively high synergic effect was seen in MDA-MB-453 cells when Smac-mimic was used in combination with TRAIL, but not etoposide. In these breast cancer cell lines, the results indicated that cellular sensitivity to Smac-mimic alone or in combination with TRAIL or etoposide was directly correlated with their endogenous levels of IAPs; the higher the levels of IAPs the more sensitive the cells became. After identifying MDA-MB-231 as a Smac-mimic sensitive breast cancer line, we then focused our mechanistic study of drug effect on this particular cell line.

Smac-mimic is a very potent cytotoxic compound in MDA-MB-231 cells:

In contrast to a short-term (19 hr drug treatment) apoptosis assay in glioblastoma T98G cells, in which Smac-mimic alone did not induce apoptosis at high concentration of >1 μM (5), the result of our 5-day MTS assay did indicate that Smac-mimic alone is a quite potent compound that can suppress MDA-MB-231 cell growth at low nanomolar concentrations. To precisely determine the IC50 value of the compound in growth suppression of this sensitive breast cancer cell line, we next used an ATPlite luminescence assay. The assay is a highly sensitive ATP monitoring system, based upon firefly luciferase for the quantitative evaluation of proliferation and cytotoxicity of cultured cells and has been used by other investigators for drug screening (6). Cells were treated with Smac-mimic alone in a concentration range from 0.1-100 nM for 24 hours (instead of 5 days), followed by an ATPlite assay to determine cell viability, which is directly correlated to the luciferase reading. Smac-mimic induced a dose dependent growth suppression with an IC50 of 3.8 nM, indicating that it is very potent as a single agent against sensitive MDA-MB-231 breast cancer cells with high IAPs expression.

Smac-mimic induces apoptosis in MDA-MB-231 cells alone and sensitizes apoptosis induced by TRAIL or etoposide:

Growth suppression, seen in Figures 2A and 3, can in theory result from growth arrest, apoptosis, or both. Since Smac-mimic has been shown to target IAPs (5), induction of apoptosis is likely the underline mechanism. To determine this, we treated cells with TRAIL, etoposide, and Smac-mimic both alone and in combination for 24 hours. Morphological observation showed that cells became shrunken and detached (signs of apoptosis) when exposed to as little as 2.5 nM Smac-mimic. A significant increase in the number of shrunken/detached cells was seen in combinational treatments with TRAIL or etoposide. Few apoptotic cells were observed due to treatment with either TRAIL or etoposide, in the concentrations used.

To further confirm that drug treatment induced apoptosis, we performed a gel-based assay to visualize DNA fragmentation, a hall-mark of apoptosis. DMSO treatment of MDA-MB-231 cells did not induce any DNA fragmentation. No obvious DNA fragmentation was observed in cells treated with TRAIL (30 ng/ml) or etoposide (5 μM) alone. However, at a concentration as low as 2.5 nM, Smac-mimic significantly induced formation of DNA fragmentation, which was further enhanced by a combinational treatment with TRAIL, but to a lesser degree with etoposide. In contrast, a 200-fold higher concentration of Smac-mimic (500 nM) did not induce DNA fragmentation in resistant T47D cells. Neither TRAIL alone (8-fold higher at 250 ng/ml, lane 2), etoposide alone (3-fold higher at 15 μM), nor a combination of Smac-mimic and TRAIL induced DNA fragmentation. A slight induction of

DNA fragmentation was seen in the combinational treatment of Smac-mimic and etoposide. The results of these two cell lines further indicate that, the induction of DNA fragmentation appears to correlate with the level of IAPs.

Finally, to quantify the degree of Smac-mimic induced apoptosis, we performed a FACS analysis. The control (DMSO) treatment induced a background level of 13% apoptosis, TRAIL (30 ng/ml) had no effect; however, etoposide (5 μM) and Smac-mimic (2.5 nM) treatments each induced about 36% apoptosis. Combinations of Smac-mimic with TRAIL or etoposide increased apoptotic cell population to 42% or 67%, respectively. Although the sensitive FACS analysis did not reach a 100% correlation with the gel-based DNA fragmentation assay, the results did show that Smac-mimic can sensitize breast cancer cells to TRAIL or etoposide and that it can even induce apoptosis at a concentration of 2.5 nM by itself.

Induction of apoptosis by Smac-mimic is through the activation of caspases:

Having established that Smac-mimic induced apoptosis alone and sensitized apoptosis induced by TRAIL or etoposide, we went on to determine whether the mechanism involved caspases activation by a caspase 3 activity assay. Drug alone treatment of MDA-MB-231 cells induced a dose-dependent increase of caspase-3 activity with TRAIL up to 2-fold, with etoposide up to 4-fold, and with Smac-mimic up to 8-fold. A weak synergic effect was seen in combinational treatments of Smac-mimic and TRAIL with a 10-fold induction of caspase-3 activity. Most significantly, the strong synergic effect of the Smac-mimic and etoposide combination yielded up to a 20-fold induction of caspase-3 activity.

Finally, we performed western blotting analyses to determine whether caspase-3 and caspase-7, two effector caspases, were activated by Smac-mimic alone or in combination with TRAIL or etoposide. Treatment with TRAIL or etoposide alone did not induce visible activation of caspases, indicated by a decreasing density of procaspase bands. Treatment with Smac-mimic alone caused a slight reduction of both procaspase-7 and procaspase-3 bands, indicating low levels of caspases activation. When used in combination with TRAIL or etoposide, Smac-mimic significantly induced activation of both caspase-7 and caspase-3, indicated by a significant reduction in the density of both bands. Similarly, cleaved PARP, a sign of caspase-3 activation and apoptosis, was not detectable when cells were treated with etoposide alone. It was shown as a faint band when cells were treated with TRAIL alone, indicating a weak activation and a more obvious band when treated with Smac-mimic alone, indicating a strong activation. Combinational treatments of Smac-mimic with either TRAIL or etoposide increased the amount of cleaved PARP product, indicating an enhanced activation of caspases and apoptosis.

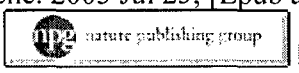
Key Research Accomplishments:

1. A recently discovered Smac-mimic compound (5) showed a low nanomolar IC50 against a breast cancer cell line that expresses high levels of IAP
2. The compound at the low nanomolar concentration also significantly sensitizes apoptosis induced by TRAIL and etoposide, two well-known agents that activate either the death-receptor (TRAIL) or mitochondrial (etoposide) apoptosis pathways in a high IAPs expressing breast cancer cell line.
3. Two other breast cancer cell lines that have low levels of IAPs expression are significantly less sensitive to this compound, further indicating that the sensitivity of these three breast cancer cells to Smac-mimic is largely determined by their cellular levels of IAPs.
4. Our study further substantiates that IAPs are truly promising targets for inducing apoptosis in a variety of human cancer cells when used in combination with other anti-cancer agents.
5. Overall, the fact that Smac-mimic at a low nanomolar concentration activates caspase-3 and induces apoptosis both in the absence and the presence of caspase activators, makes it very appealing for future development as a novel anti-cancer drug.

Reportable outcomes:

1) This work is now published in Oncogene

Oncogene. 2005 Jul 25; [Epub ahead of print] Related Articles, Links



A small molecule Smac-mimic compound induces apoptosis and sensitizes TRAIL- and etoposide-induced apoptosis in breast cancer cells.

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Department of Radiation Oncology, Division of Cancer Biology, University of Michigan Comprehensive Cancer Center, 4304 CCGC, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0936, USA.

2) The first author presented this work as a symposium platform presentation at the Era of Hope Department of Defense Breast Cancer Research Program Meeting. June 8-11, 2005.

Conclusion:

IAPs are truly promising targets for inducing apoptosis in a variety of human cancer cells when used in combination with other anti-cancer agents. The fact that this Smac-mimic small molecule at a low nanomolar concentration activates caspase-3 and induces apoptosis both in the absence and the presence of caspase activators, makes it very appealing for future development as a novel anti-cancer drug.

References

1. Deveraux, Q. L. and Reed, J. C. IAP family proteins--suppressors of apoptosis. Genes Dev, *13:* 239-252, 1999.
2. Chai, J., Du, C., Wu, J. W., Kyin, S., Wang, X., and Shi, Y. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. Nature, *406:* 855-862., 2000.
3. Wu, G., Chai, J., Suber, T. L., Wu, J. W., Du, C., Wang, X., and Shi, Y. Structural basis of IAP recognition by Smac/DIABLO. Nature, *408:* 1008-1012., 2000.
4. Arnt, C. R., Chiorean, M. V., Heldebrant, M. P., Gores, G. J., and Kaufmann, S. H. Synthetic Smac/DIABLO peptides enhance the effects of chemotherapeutic agents by binding XIAP and cIAP1 in situ. J Biol Chem, *277:* 44236-44243., 2002.
5. Li, L., Thomas, R. M., Suzuki, H., De Brabander, J. K., Wang, X., and Harran, P. G. A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. Science, *305:* 1471-1474, 2004.
6. Boyce, M., Bryant, K. F., Jousse, C., Long, K., Harding, H. P., Scheuner, D., Kaufman, R. J., Ma, D., Coen, D. M., Ron, D., and Yuan, J. A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. Science, *307:* 935-939, 2005.

Appendices

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ORIGINAL PAPER

A small molecule Smac-mimic compound induces apoptosis and sensitizes TRAIL- and etoposide-induced apoptosis in breast cancer cells

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Inhibitor of apoptosis protein (IAP) suppresses apoptosis through binding and inhibiting active caspases-3, -7 and -9 via its baculoviral IAP repeat (BIR) domains. During apoptosis the caspase inhibition by IAPs can be negatively regulated by a mitochondrial protein second mitochondrial-derived activator of caspase (Smac). Smac physically interacts with multiple IAPs and relieves their inhibitory effect on caspases-3, -7 and -9. Recently, a small molecule Smac-mimic compound (Smac-mimic), which potentiates TNF-related apoptosis-inducing ligand (TRAIL) and tumor necrosis factor (TNF)-α mediated cell death in glioblastoma T98G cells and HeLa cells, was identified and characterized. To determine the efficacy of this compound in breast cancer cells, we first measured protein expression of three IAPs: XIAP, cIAP-1, and cIAP-2 in nine independent breast cancer cell lines. Three cell lines were chosen: a high IAPs expressing line MDA-MB-231, and two low IAPs expressing lines, T47D and MDA-MB-453. The cell lines were tested for their sensitivity to Smac-mimic alone or in combination with TRAIL or etoposide. Acting alone, Smac-mimic was quite potent with a cytotoxic IC_{50} of 3.8 nM in high IAPs expressing MDA-MB-231 cells, but was inactive at a much higher concentration in low IAPs expressing T47D and MDA-MB-453 cells. In fact, as low as 2.5 nM of Smac-mimic alone was sufficient to activate caspase-3 and induce apoptosis in MDA-MB-231 cells. In combinational treatments with TRAIL or etoposide, Smac-mimic significantly sensitized cells to growth suppression in MDA-MB-231 cells, but to a lesser extent in T47D and MDA-MB-453 cells. Furthermore, it significantly synergized MDA-MB-231, but not T47D cells to apoptosis induced by either TRAIL or etoposide. Thus, in these cell lines, Smac-mimic acts in an apparent IAPs dependent manner to induce apoptosis alone as well as sensitizes breast cancer cells to TRAIL or etoposide induced apoptosis via caspase-3 activation.
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Introduction

Apoptosis is a genetically programmed process of cell death required for maintaining homeostasis under physiological conditions and for responding to various internal and external stimuli (Kerr, 1971; Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Thompson, 1995). Cells committed to apoptosis are characterized by membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation and DNA fragmentation (Wyllie, 1980). Cancer is one of the human diseases that is associated with decreased apoptosis (Thompson, 1995) and development of apoptosis resistance in cancer cells is a significant contributing factor to the failure of cancer therapies (Makin and Hickman, 2000). Thus, induction of apoptosis in apoptosis-resistant cancer cells through a variety of approaches would be an ideal strategy for effective cancer therapy (Nguyen and Wells, 2003; Reed, 2003).

Two major signaling pathways leading to apoptosis-associated caspase activation were defined in mammalian cells: the extrinsic death-receptor pathway that is triggered by members of the death receptor superfamily, leading to caspase-8 activation; and the intrinsic mitochondrial pathway that is activated in response to extra-cellular cues and internal insults, such as DNA damage, resulting in cytochrome *c* release and caspase-9 activation (Hengartner, 2000). Both the intrinsic and extrinsic pathways for caspase activation converge on caspase-3 whose activation commits cells to apoptosis (Earnshaw *et al.*, 1999; Wolf *et al.*, 1999).

One of the well-characterized apoptosis inhibitor families is inhibitor of apoptosis protein (IAP), which consists of at least eight family members (Deveraux and Reed, 1999; Salvesen and Duckett, 2002). IAP family proteins are characterized by containing one or several baculovirual IAP repeat (BIR) domains, which are required for suppression of apoptosis. Some family members also have a RING-finger domain at the C-terminus that is required for ubiquitination and degradation of caspases (Deveraux and Reed, 1999; Yang and Li, 2000). The main function of IAPs is to suppress apoptosis via binding and inhibiting active caspases-3, -7 and -9. The most potent inhibitor among IAP family members is XIAP with subnanomolar inhibitory constants against caspases (Deveraux and Reed, 1999). In XIAP, the third BIR domain (BIR3)

potently inhibits the activity of the active caspase-9 whereas the linker region between BIR1 and BIR2 selectively targets the active caspase-3 and -7 (Sun *et al.*, 1999; Chai *et al.*, 2001; Huang *et al.*, 2001; Riedl *et al.*, 2001; Srinivasula *et al.*, 2001). A two-binding-site interaction mechanism has been recently demonstrated by which XIAP inhibits caspase-3 and -7 (Scott *et al.*, 2005).

In apoptotic cells, the caspase inhibition by IAPs is negatively regulated by a mitochondrial protein called Smac. Smac physically interacts with multiple IAPs and relieves their inhibitory effect on active caspases-3, -7 and -9 (Chai *et al.*, 2000). The crystal structure showed that this is mainly achieved through the N-terminal four conserved residues (AVPI) in a mature Smac molecule that recognizes and binds to a surface groove on the BIR3 domain of XIAP (Wu *et al.*, 2000). Indeed, synthetic Smac peptides with AVPI in their N-terminus are sufficient to activate caspase-3 (Chai *et al.*, 2000; Wu *et al.*, 2000) and sensitize breast cancer cells to apoptosis induced by chemotherapeutic agents (Arnt *et al.*, 2002). Since the molecular weight of tetrapeptide AVPI is quite small and the structure of its binding to XIAP is available, it is feasible to set up a binding assay to screen for small molecules that compete with the tetrapeptides for XIAP binding, thus activating caspases and inducing apoptosis. Indeed, such a small molecule Smac-mimic compound has been identified and shown to be active in sensitizing TRAIL- and TNF-α-mediated cell death in glioblastoma T98G cells and HeLa cells (Li *et al.*, 2004). We report here that this Smac-mimic compound is very potent in inducing apoptosis in the tested breast cancer cells alone or in combination with TRAIL or etoposide in a manner dependent upon cellular levels of IAPs.

Results

Expression of IAPs varies among breast cancer cell lines

A newly identified Smac-mimic small molecule compound (Smac-mimic) acts by targeting IAPs to relieve their suppression of caspase activity (Li *et al.*, 2004). This mechanism of action suggests that Smac-mimic could have a higher efficacy in inducing apoptosis in cancer cells with over-expressed IAPs. To determine efficacy of Smac-mimic in breast cancer cells and to establish a potential correlation between levels of IAPs and drug sensitivity, a total of nine breast cancer cell lines were examined by Western blotting analysis for their protein levels of three well-studied IAPs: XIAP, cIAP-1 and cIAP-2. As shown in Figure 1, expression of IAPs varies significantly among these lines. In general, most of the cell lines tested have high or detectable expression of XIAP and cIAP-1, but low expression of cIAP-2. Based upon the expression levels of IAPs, three cell lines were selected to determine their sensitivity to Smac-mimic: MDA-MB-231 (lane 2) a high IAPs expresser, with high levels of XIAP and cIAP-1, but low levels of cIAP-2; MDA-MB-453 (lane 3) a low IAPs expresser, with no or very low levels of cIAP-1 and XIAP, but relatively high levels of cIAP-2; and T47D (lane 4) another low IAPs expresser with low levels of all three IAPs.

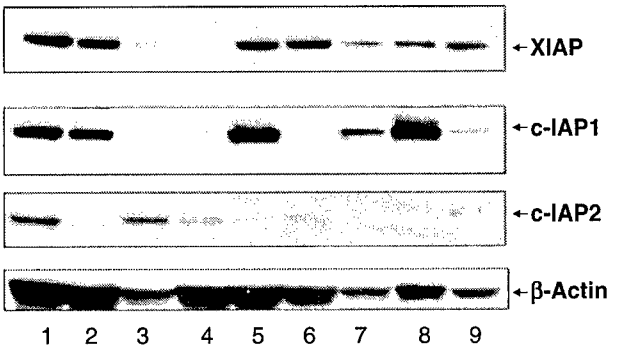

Figure 1 Expression of IAPs in multiple human breast cancer cells. A total of nine breast cancer cell lines (lane 1: MDA-MB-468; lane 2: MDA-MB-231; lane 3: MDA-MB-453; lane 4: T47D; lane 5: ZR75-1; lane 6: MCF7; lane 7: SUM149; lane 8: SUM102; and lane 9: SUM52) were grown in DMEM supplemented with 10% FBS. Subconfluent cells were harvested and subjected to Western analysis using antibodies against XIAP, c-IAP1 and c-IAP2 as indicated. β-Actin was used as protein loading control

Levels of endogenous IAPs determine cellular sensitivity to Smac-mimic alone or in combination with TRAIL or etoposide

We next determined, using a 5-day cell proliferation MTS assay, the sensitivity of these three cell lines to Smac-mimic, TRAIL (a ligand for TRAIL receptors to initiate the death receptor apoptosis pathway), or etoposide (a DNA-damaging agent to activate the mitochondrial-initiated apoptosis pathway) alone and in combination. The drug concentration used was predetermined so that it did not induce significant growth suppression when used alone, thus a synergic effect of the drug combinations can be seen. As shown in Figure 2a, in high IAPs expressing MDA-MB-231 cells, TRAIL and etoposide alone at a concentration of 50 ng/ml and 500 nM, respectively, induced 10–15% growth inhibition (85–90% cell viability, compared to DMSO control), whereas Smac-mimic caused 40% inhibition at a concentration of 10 nM. When used in combination, Smac-mimic and TRAIL caused up to 100% growth suppression, whereas Smac-mimic and etoposide inhibited growth up to 80%. These results indicated that not only was there a synergic effect of Smac-mimic on agents that activate either death receptor or mitochondrial apoptosis pathways, but Smac-mimic alone had some effect.

In contrast, both low IAPs expressing cell lines, T47D (Figure 2b) and MDA-MB-453 (Figure 2c) were quite resistant to these drugs when given alone, although in T47D cells etoposide did inhibit cell growth by 40%. In the case of Smac-mimic, fivefold higher drug concentration (50 nM) did not induce any growth inhibition in either low IAPs expressing cell line. Combinations of Smac-mimic at this high concentration

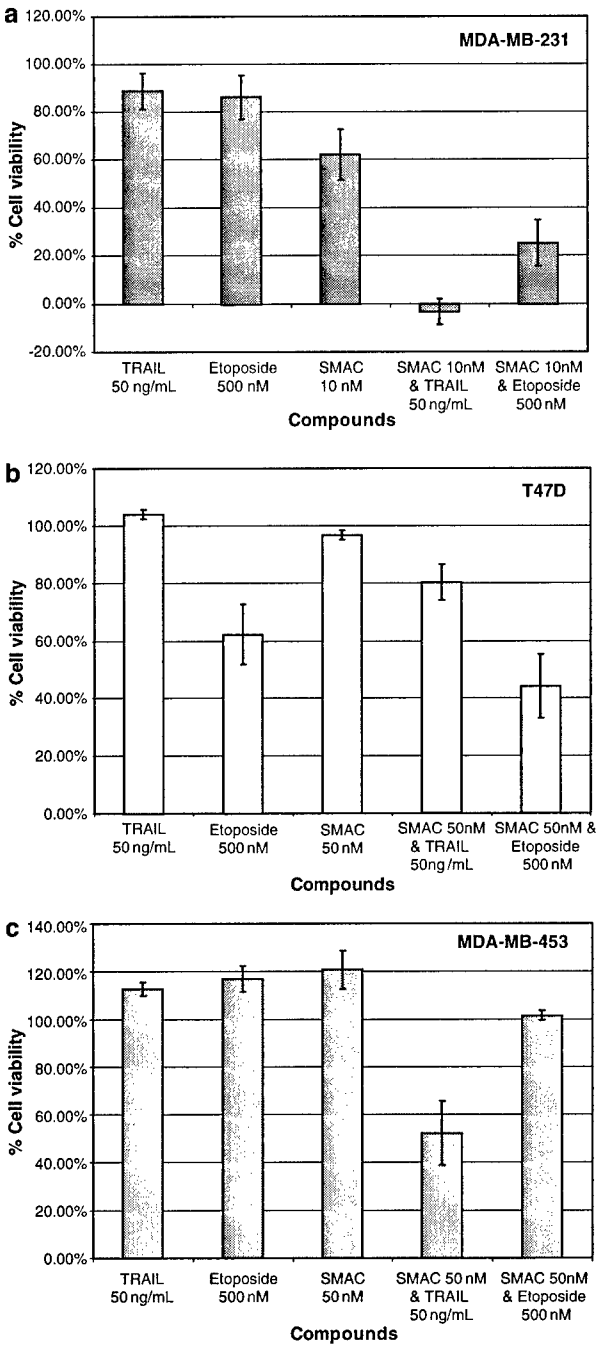

Figure 2 Growth inhibition of breast cancer cells by Smac-mimic compound alone or in combination with TRAIL or etoposide. Cells from the high IAPs expressing MDA-MB-231 line (**a**) and two low IAPs expressing lines, T47D (**b**) and MDA-MB-453 (**c**) were seeded in 96-well plates (3000 cells/well for MDA-MB-231 and T47D, and 6000 cells/well for MDA-MB-453) on day 1. On day 2, cells were subjected to the treatment of DMSO (control), TRAIL, etoposide, or Smac-mimic compound (Smac-mimic) alone or in combination with indicated concentrations. Cell viability was measured after 5 days drug treatment using a MTS cell proliferation assay. Shown is the mean$\pm$s.e.m. of percent cell viability, as compared to DMSO control, from three independent assays, each run had five wells per treatment

with TRAIL or etoposide gave rise to a moderate synergic effect in T47D. A relatively high synergic effect was seen in MDA-MB-453 cells when Smac-mimic was used in combination with TRAIL, but not etoposide. In these breast cancer cell lines, the results indicated that cellular sensitivity to Smac-mimic alone or in combination with TRAIL or etoposide was directly correlated with their endogenous levels of IAPs; the higher the levels of IAPs the more sensitive the cells became. After identifying MDA-MB-231 as a Smac-mimic-sensitive breast cancer line, we then focused our mechanistic study of drug effect on this particular cell line.

Smac-mimic is a very potent cytotoxic compound in MDA-MB-231 cells

In contrast to a short-term (19 h drug treatment) apoptosis assay in glioblastoma T98G cells, in which Smac-mimic alone did not induce apoptosis at high concentrations of $>1\,\mu$M (Li *et al.*, 2004), the result of our 5-day MTS assay, shown in Figure 2a, did indicate that Smac-mimic alone is a quite potent compound that can suppress MDA-MB-231 cell growth at low nanomolar concentrations. To precisely determine the IC_{50} value of the compound in growth suppression of this sensitive breast cancer cell line, we next used an ATPlite luminescence assay. The assay is a highly sensitive ATP monitoring system, based upon firefly luciferase for the quantitative evaluation of proliferation and cytotoxicity of cultured cells and has been used by other investigators for drug screening (Boyce *et al.*, 2005). Cells were treated with Smac-mimic alone in a concentration range from 0.1 to 100 nM for 24 h (instead of 5 days), followed by an ATPlite assay to determine cell viability, which is directly correlated to the luciferase reading. The results are presented as percentage of cell viability compared to DMSO control. As shown in Figure 3, Smac-mimic induced a dose dependent growth suppression with an IC_{50} of 3.8 nM, indicating that it is very potent as a single agent against sensitive MDA-MB-231 breast cancer cells with high IAPs expression.

Smac-mimic induces apoptosis in MDA-MB-231 cells alone and sensitizes apoptosis induced by TRAIL or etoposide

Growth suppression, seen in Figures 2a and 3, can in theory result from growth arrest, apoptosis, or both. Since Smac-mimic has been shown to target IAPs (Li *et al.*, 2004), induction of apoptosis is likely the underline mechanism. To determine this, we treated cells with TRAIL, etoposide, and Smac-mimic both alone and in combination for 24 h. Morphological observation showed that cells became shrunken and detached (signs of apoptosis) when exposed to as little as 2.5 nM Smac-mimic (Figure 4A, panel d). A significant increase in the number of shrunken/detached cells was seen in combinational treatments with TRAIL (panel e) or etoposide (panel f). Few apoptotic cells were observed due to treatment with either TRAIL (panel b) or etoposide (panel c), at the concentrations used.

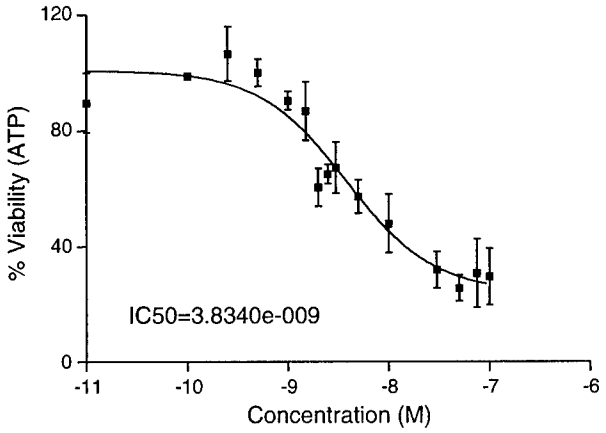

Figure 3 Smac-mimic compound inhibited growth of MDA-MB-231 cells at low nanomolar concentration. Cells were seeded in 96-well plates at a density of 1.5×10^4 cells per well on day 1 and treated on day 2 with Smac-mimic in a dose range from 0.1 to 100 nM. After 24 h of treatment, cells were subjected to ATPlite assay for cell viability. Results were expressed as percent viability ± s.e.m., compared to DMSO control, from four independent experiments, each having three wells per drug concentration. The curve was generated using PRISM with the IC_{50} calculated by the software

To further confirm that drug treatment-induced apoptosis, we performed a gel-based assay to visualize DNA fragmentation, a hall-mark of apoptosis. DMSO treatment of MDA-MB-231 cells did not induce any DNA fragmentation (data not shown). No obvious DNA fragmentation was observed in cells treated with TRAIL (30 ng/ml) or etoposide (5 μM) alone (Figure 4B, left panel, lanes 1 and 2). However, at a concentration as low as 2.5 nM, Smac-mimic significantly induced formation of DNA fragmentation (lane 3), which was further enhanced by a combinational treatment with TRAIL (lane 4), but to a lesser degree with etoposide (lane 5). In contrast, a 200-fold higher concentration of Smac-mimic (500 nM) did not induce DNA fragmentation in resistant T47D cells (Figure 4B, right panel, lane 4). Neither TRAIL alone (eightfold higher at 250 ng/ml, lane 2), etoposide alone (threefold higher at 15 μM, lane 3), nor a combination of Smac-mimic and TRAIL (lane 5) induced DNA fragmentation. A slight induction of DNA fragmentation was seen in the combinational treatment of Smac-mimic and etoposide (lane 6). The results of these two cell lines further indicate that, the induction of DNA fragmentation appears to correlate with the level of IAPs.

Finally, to quantify the degree of Smac-mimic induced apoptosis, we performed a FACS analysis; the data is presented in Figure 4C. The control (DMSO) treatment induced a background level of 13% apoptosis, TRAIL (30 ng/ml) had no effect; however, etoposide (5 μM) and Smac-mimic (2.5 nM) treatments each induced about 36% apoptosis. Combinations of Smac-mimic with TRAIL or etoposide increased apoptotic cell population to 42 or 67%, respectively. Although the sensitive FACS analysis did not reach a 100% correlation with the gel-based DNA fragmentation assay, the results did show that Smac-mimic can sensitize breast cancer cells to TRAIL or etoposide and that it can even induce apoptosis at a concentration of 2.5 nM by itself.

Induction of apoptosis by Smac-mimic is through the activation of caspases

Having established that Smac-mimic induced apoptosis alone and sensitized apoptosis induced by TRAIL or etoposide, we went on to determine whether the mechanism involved caspases activation by a caspase-3 activity assay. As shown in Figure 5a, drug alone treatment of MDA-MB-231 cells induced a dose-dependent increase of caspase-3 activity with TRAIL up to twofold, with etoposide up to fourfold, and with Smac-mimic up to eightfold. A weak synergic effect was seen in combinational treatments of Smac-mimic and TRAIL with a 10-fold induction of caspase-3 activity. Most significantly, the strong synergic effect of the Smac-mimic and etoposide combination yielded up to a 20-fold induction of caspase-3 activity.

Finally, we performed Western blotting analyses to determine whether caspase-3 and -7, two effector caspases, were activated by Smac-mimic alone or in combination with TRAIL or etoposide. As shown in Figure 5b, treatment with TRAIL or etoposide alone did not induce visible activation of caspases, indicated by a decreasing density of procaspase bands (top two panels, lanes 2 and 3, compared to lane 1). Treatment with Smac-mimic alone caused a slight reduction of both procaspase-7 and procaspase-3 bands (lane 4), indicating low levels of caspases activation. When used in combination with TRAIL or etoposide, Smac-mimic significantly induced activation of both caspase-7 and -3, indicated by a significant reduction in the density of both bands (lanes 5 and 6). Similarly, cleaved PARP, a sign of caspase-3 activation and apoptosis, was not detectable when cells were treated with etoposide alone (lane 3). It was shown as a faint band when cells were treated with TRAIL alone (lane 2), indicating a weak activation and a more obvious band when treated with Smac-mimic alone (lane 4), indicating a strong activation. Combinational treatments of Smac-mimic with either TRAIL or etoposide increased the amount of cleaved PARP product, indicating an enhanced activation of caspases and apoptosis.

Discussion

Although facing enormous uncertainty in predicting sound preclinical activity in human efficacy, discovery and development of apoptosis-based novel anticancer drugs has advanced rapidly with few promising agents reaching phase I clinical trials (Garber, 2005). One appealing target is the IAPs family, which binds to both an initiator caspase (caspase-9) and effector caspases (caspase-3 and -7) to block caspase-dependent apop-

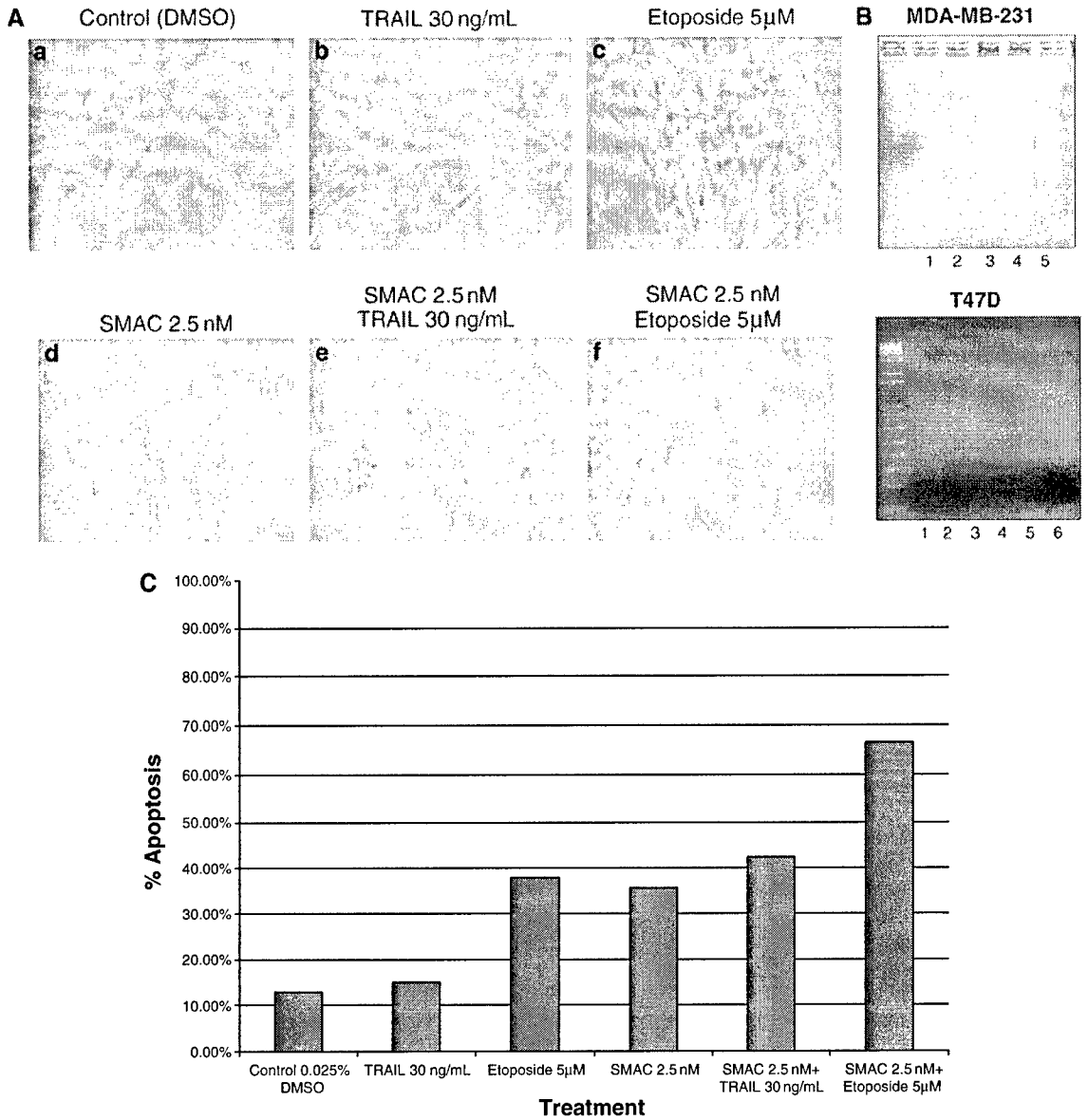

Figure 4 Smac-mimic compound-induced apoptosis in MDA-MB-231 cells at low nanomolar concentrations alone or in combination with TRAIL or etoposide. **(A)** Morphological observation of apoptotic cells: MDA-MB-231 cells were seeded in 60 mm dishes (9×10^5 cells) on day 1 and subjected to drug treatments at indicated concentrations on day 2 for 24 h. Cellular morphology was observed in a light microscope with a magnification of $\times 200$. **(B)** Induction of DNA fragmentation: MDA-MB-231 cells or T47D cells were seeded in 100 mm dishes at densities of 2.5×10^6 or 4×10^6 cells per dish, respectively. The next day, cells were treated for 24 h with DMSO control (lane 1 for T47D, not shown in MDA-MB-231); TRAIL (250 ng/ml for T47D, lane 2; or 30 ng/ml for 231, lane 1); etoposide (15 μM for T47D, lane 3; or 5 μM for 231, lane 2); Smac-mimic (500 nM for T47D, lane 4; or 2.5 nM for 231, lane 3); Smac-mimc and TRAIL (T47D, lanc 5; 231, lanc 4); or Smac-mimic and etoposide (T47D, lanc 6; 231, lanc 5). Both detached and attached cells were harvested by scraping and subjected to gel-based DNA fragmentation. A 1 kb plus size marker is shown on the left. **(C)** Apoptosis induction quantified by FACS analysis: MDA-MB-231 cells were seeded in 60 mm dishes (9×10^5 cells) on day 1 and subjected to drug treatments at indicated concentrations on day 2 for 24 h. Cells were fixed in 70% ethanol and frozen at -20°C for at least 4 h, suspended in 1 × Propidium Iodide solution with 400 μg/ml RNase (Roche), and analysed in the Flow Cytometry Lab facility at the University of Michigan. The percent apoptosis is the percent of cells in the sub G_1 population

tosis. IAPs have been validated as a cancer target in many preclinical studies with a variety of approaches. For example, targeting XIAP or survivin via RNAi and antisense sensitized cancer cells to a variety of chemotherapeutic reagents as well as radiation (Yang *et al*., 2003a; Cao *et al*., 2004; Lima *et al*., 2004; McManus *et al*., 2004). Synthetic Smac peptides, with AVPI in their N-terminus, fused to cell-permeablizing peptides have been shown to sensitize both human cancer cells (in culture) and tumor xenografts (in mice) to apoptosis induced by TRAIL or chemotherapeutic drugs, by disrupting IAP-caspases binding (Arnt *et al*.,

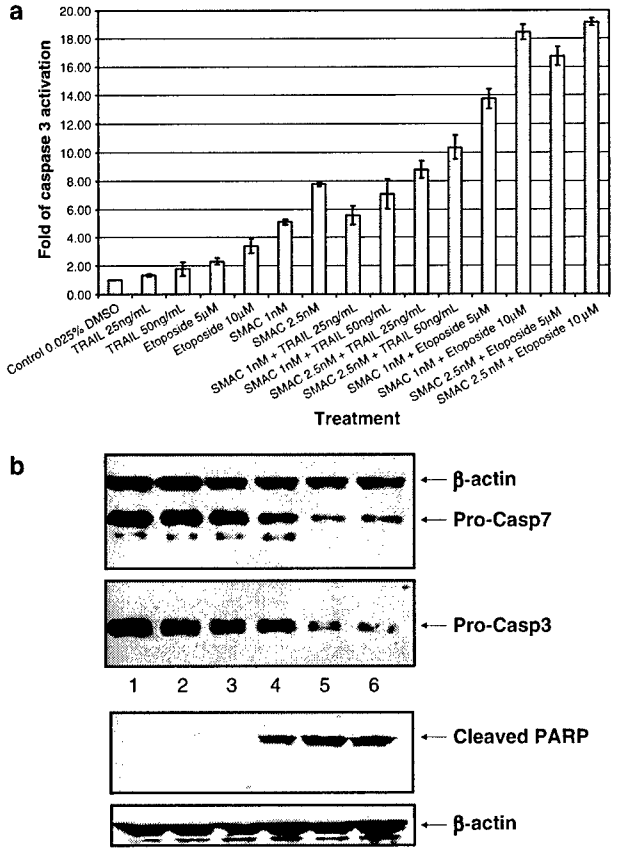

Figure 5 Smac-mimic compound activated caspase-3 alone or in combination of TRAIL and etoposide. (**a**) Activation of caspase-3 activity: MDA-MB-231 cells were seeded in 96-well plates at a density of 1.5×10^4 cells per well. The next day, cells were treated with DMSO (control), TRAIL, etoposide, or Smac-mimic alone or in combination as indicated for 24 h, followed by caspase-3 activity assay using Ac-DEVD-AFC as the fluorogenic substrate. Shown is the mean $\pm$ s.e.m. of fold-activation of caspase-3 activity from three independent assays; each sample was run in triplicate and compared to DMSO control, which is set at 1. (**b**) Activation of pro-caspases and PARP cleavage: MDA-MB-231 cells were seeded in 100 mm dishes at 2×10^6 cells per dish. Cells were cultured for 24 h and subjected to treatment with DMSO (control, lane 1), TRAIL (50 ng/ml, lane 2), etoposide (3 μM, lane 3), Smac-mimic (5 nM, lane 4), Smac-mimic and TRAIL (lane 5) or Smac-mimic and etoposide (lane 6) for 24 h. The cells were harvested and the cell lysate was prepared and subjected to Western blotting analysis, using antibodies against PARP, caspase-3, and -7. β-Actin served as loading control

2002; Fulda *et al*., 2002; Pardo *et al*., 2003; Yang *et al*., 2003b).

In this study, we showed that the recently discovered Smac-mimic compound (Li *et al*., 2004) at low nanomolar concentrations significantly sensitizes apoptosis induced by TRAIL and etoposide, two well-known agents that activate either the death-receptor (TRAIL) or mitochondrial (etoposide) apoptosis pathways in a high IAPs expressing breast cancer cell line. Two other breast cancer cell lines that have low levels of IAPs expression are significantly less sensitive to this compound, further indicating that the sensitivity of these three breast cancer cells to Smac-mimic is largely determined by their cellular levels of IAPs. The compound's high potency is derived from its ability to mimic Smac protein's dimeric structure, which acts at both the BIR3 and BIR2-linker regions, releasing inhibitory binding of IAPs to active caspases-3, -7 and -9 (Li *et al*., 2004). Our study further substantiates that IAPs are truly promising targets for inducing apoptosis in a variety of human cancer cells when used in combination with other anticancer agents.

In contrast to T98G and HeLa cells that are resistant to Smac-mimic, when given alone (Li *et al*., 2004), MDA-MB-231 breast cancer cells with high levels of IAPs are quite sensitive to Smac-mimic with a cytotoxic IC_{50} of 3.8 nM. As little as 2.5 nM Smac-mimic is sufficient to induce substantial caspase-3 activation and apoptosis. The fact that two other breast cancer cell lines with lower levels of IAPs are resistant to the compound suggests an IAPs-dependent effect, although other cell line dependent contributing 'factors' cannot be excluded. It is well known that XIAP binds to active forms of caspase-3, -7 and -9 (Sun *et al*., 1999; Chai *et al*., 2001; Huang *et al*., 2001; Riedl *et al*., 2001; Srinivasula *et al*., 2001). In high IAPs expressing cells, such as MDA-MB-231 cells, the IAPs are the major defense against apoptosis induced by caspases activation. Once Smac-mimic releases the basal level of active caspases, they in turn activate the procaspases in a feed-forward self-amplifying manner. This process is accelerated in the presence of other caspases activating agents such as etoposide or TRAIL (Figure 5b). Consistent with our observation, it has been previously shown that adenoviral infection of Smac alone induced apoptosis in a dose dependent manner in ovarian carcinoma cells (McNeish *et al*., 2003). A recent study also showed that, when used as a single agent, several capped tripeptide antagonists, which specifically target the BIR3 domain of XIAP, promoted cell death in several human cancer cell lines and inhibited *in vivo* growth of MDA-MB-231 breast cancer cells in nude mice (Oost *et al*., 2004). Overall, the fact that Smac-mimic at a low nanomolar concentration activates caspase-3 and induces apoptosis both in the absence and the presence of caspase activators, makes it very appealing for future development as a novel anticancer drug.

Materials and methods

Compound

Smac-mimic was a gift from Dr Xiaodong Wang at the Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern Medical Center.

Cell culture

Six breast cancer cell lines: MDA-MB-468, MDA-MB-231, MDA-MB-453, T47D, ZR75-1, and MCF7 were obtained from ATCC. The other three breast cancer cell lines: SUM149, SUM102 and SUM52 were obtained as the gift from

Dr Stephen Ethier. All lines were grown in DMEM containing 10% FBS.

Western blotting analysis

For determination of IAPs expression in breast cancer cell lines, subconfluent cells were harvested and lysed in a Titron X-100 lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton, 5 mM EGTA and 5 mM EDTA) with freshly added protease inhibitor tablet for 1 h on ice, then centrifuged for 30 min. Supernatants were measured for protein concentration using a Bio-Rad protein assay reagent (Bio-Rad) and subjected to Western blotting (Duan *et al*., 1999) using antibodies against XIAP, c-IAP1 and c-IAP2 (Cell Signaling). As a control, the same membranes were stripped and immunoblotted again with anti-β-actin antibody (Sigma).

For Western blotting analysis of caspases activation, the cells were seeded in 100 mm dishes at $\sim 2 \times 10^6$ cells per dish. On day 2, the cells were treated with DMSO (control), 50 ng/ml TRAIL, 3 μM etoposide, 5 nM Smac-mimic, or combinations of Smac-mimic and TRAIL and Smac-mimic and etoposide for 24 h. The cells were then lysed, subjected to immunoblotting using antibodies against caspase-3, -7 (BD Biosciences) or PARP (Cell Signaling). Both were also probed against β-Actin (Sigma), which was used as a loading control.

Five-day cell viability assay

The cells were seeded in 96-well plates overnight; MDA-MB-231 and T47D at 3000 cells per well and MDA-MB-453 at 6000 cells per well. The cells were then treated with 50 ng/ml TRAIL, 500 nM etoposide, 10 nM Smac-mimic for MDA-MB-231 or 50 nM Smac-mimic for T47D and MDA-MB-453 cells, or combinations of Smac-mimic and TRAIL and Smac-mimic and etoposide. DMSO was used as a control. After 5 days of treatment, the viability of the cells was measured using a MTS kit (Promega) as per the manufacturer's instructions. The plates were read 2 h after the addition of the MTS solutions in a Beckman Coulter LD 400 at 492 nM. The results are seen as percent survival compared to control. Each treatment had five replicates.

ATPlite cell viability and IC_{50} assay

MDA-MB-231 cells were seeded in 96-well plates at 1.5×10^4 cells per well. On day 2, the cells were treated with Smac-mimic dilutions from 0.1 to 100 nM for 24 h. The viability of the cells was then measured using an ATPlite kit (Perkin Elmer) following the manufacturer's instructions with a few modifications. The cells were cultured in DMEM without phenol red (Invitrogen) and the lysates were transferred from regular transparent tissue culture treated plates to white plates. After the addition of the luminescent substrate the plates were covered in foil. Before reading the plates they were allowed to dark-adapt for 5 min in the plate reader. Luminescence was read using a Beckman Coulter LD 400 plate reader. The same experiments were also carried out in tissue culture treated white plates, everything else was the same. The results, from four independent experiments with three replicates for each treatment, were then plotted in Prism (Graphpad).

DNA fragmentation analysis

The cells were seeded in 100 mm dishes at 2.5×10^6 (MDA-MB-231) or 4×10^6 (T47D) cells per dish. The next day, the cells were treated with DMSO (control), TRAIL (30 ng/ml MDA-MB-231, 250 ng/ml T47D), etoposide (5 μM MDA-MB-231, 15 μM T47D), Smac-mimic (2.5 nM MDA-MB-231, 500 nM T47D), or combinations of Smac-mimic and TRAIL and Smac-mimic and etoposide for 24 h. The cells were harvested by scraping, pelleted and lysed in 600 μl of lysis buffer (5 mM Tris-HCl, pH 8, 20 mM EDTA and 0.5% Triton X-100). The DNA was isolated using Phenol:Chloroform: Isoamine (Fisher) extraction and ethanol precipitation, and subjected to electrophoresis in a 1.8% agarose gel (Sun *et al*., 1997).

Cell morphology and FACS analysis

MDA-MB-231 cells were seeded in 60 mm dishes at 9×10^5 cells per plate. The next day, the cells were treated with DMSO (control), 30 ng/ml TRAIL, 5 μM etoposide, 2.5 nM Smac-mimic, or combinations of Smac-mimic and TRAIL and Smac-mimic and etoposide for 24 h. Photos of the cells were taken to show the morphology of the cells due to the treatments. Afterwards the cells were harvested with Trypsin (Invitrogen) and fixed in 70% EtOH at -20°C for at least 4 h, suspended in 1 × Propidium Iodide solution with 400 μg/ml RNase (Roche), and analysed in the Flow Cytometry Lab facility at the University of Michigan. The percent apoptosis is the percent of cells in the subG$_1$ population.

Caspase-3 activation assay

The activity of caspase-3 when induced by Smac-mimic, TRAIL, or etoposide alone and in combination was analyzed using a fluorogenic caspase-3 assay with Ac-DEVD-AFC as a substrate (Biomol). The cells were seeded in 96-well plates at 1.5×10^4 cells per well. On day 2, the cells were treated with DMSO (control), 25 or 50 ng/ml TRAIL, 5 or 10 μM etoposide, 1 or 2.5 nM Smac-mimic, or combinations of Smac-mimic and TRAIL and Smac-mimic and etoposide for 24 h. The cells were then lysed by adding 20 μl of a 6 × lysis buffer (10 mM HEPES pH 7.4, 42 mM KCl, 5 mM $MgCl_2$, 0.66 mM EGTA, 0.66 mM EDTA, 3.4% (w/v) CHAPS, 10 mM DTT and 1 protease inhibitor cocktail tablet (Roche) per 50 ml) into 100 μl of cell medium. The plates were shaken for 20 min, followed by adding 80 μl of a 2.5 × caspase-3 activity assay buffer (120 mM Hepes, pH 7.4, 630 mM sucrose, 0.25% CHAPS, 10 mM DTT and 125 μM Ac-DEVD-AFC fluorescent substrate). The plates were wrapped in foil and read 2 h after the addition of Ac-DEVD-AFC at 400 (excitation) and 505 (emission) using a Tecan Ultra plate reader (Tecan). The results were expressed as fold change compared to control. Each treatment had three replicates.

Abbreviations

BIR, baculovirual IAP repeat; IAPs, inhibitors of apoptosis protein; Smac, second mitochondrial-derived activator of caspase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

Acknowledgements

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References

Arnt CR, Chiorean MV, Heldebrant MP, Gores GJ and Kaufmann SH. (2002). *J. Biol. Chem.*, **277,** 44236–44243.

Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, Kaufman RJ, Ma D, Coen DM, Ron D and Yuan J. (2005). *Science*, **307,** 935–939.

Cao C, Mu Y, Hallahan DE and Lu B. (2004). *Oncogene*, **23,** 7047–7052.

Chai J, Du C, Wu JW, Kyin S, Wang X and Shi Y. (2000). *Nature*, **406,** 855–862.

Chai J, Shiozaki E, Srinivasula SM, Wu Q, Dataa P, Alnemri ES and Shi Y. (2001). *Cell*, **104,** 769–780.

Deveraux QL and Reed JC. (1999). *Genes Dev.*, **13,** 239–252.

Duan H, Wang Y, Aviram M, Swaroop M, Loo JA, Bian J, Tian Y, Mueller T, Bisgaier CL and Sun Y. (1999). *Mol. Cell. Biol.*, **19,** 3145–3155.

Earnshaw WC, Martins LM and Kaufmann SH. (1999). *Ann. Rev. Biochem.*, **68,** 383–424.

Fulda S, Wick W, Weller M and Debatin KM. (2002). *Nat. Med.*, **8,** 808–815.

Garber K. (2005). *Nat. Biotechnol.*, **23,** 409–411.

Hengartner MO. (2000). *Nature*, **407,** 770–776.

Huang Y, Park YC, Rich RL, Segal D, Myszka DG and Wu H. (2001). *Cell*, **104,** 781–790.

Kerr JF. (1971). *J. Pathol.*, **105,** 13–20.

Kerr JF, Wyllie AH and Currie AR. (1972). *Br. J. Cancer*, **26,** 239–257.

Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X and Harran PG. (2004). *Science*, **305,** 1471–1474.

Lima RT, Martins LM, Guimaraes JE, Sambade C and Vasconcelos MH. (2004). *Cancer Gene Ther.*, **11,** 309–316.

Makin G and Hickman JA. (2000). *Cell Tissue Res.*, **301,** 143–152.

McManus DC, Lefebvre CA, Cherton-Horvat G, St-Jean M, Kandimalla ER, Agrawal S, Morris SJ, Durkin JP and Lacasse EC. (2004). *Oncogene*, **23,** 8105–8117.

McNeish IA, Bell S, McKay T, Tenev T, Marani M and Lemoine NR. (2003). *Exp. Cell Res.*, **286,** 186–198.

Nguyen JT and Wells JA. (2003). *Proc. Natl. Acad. Sci. USA*, **100,** 7533–7538.

Oost TK, Sun C, Armstrong RC, Al-Assaad AS, Betz SF, Deckwerth TL, Ding H, Elmore SW, Meadows RP, Olejniczak ET, Oleksijew A, Oltersdorf T, Rosenberg SH, Shoemaker AR, Tomaselli KJ, Zou H and Fesik SW. (2004). *J. Med. Chem.*, **47,** 4417–4426.

Pardo OE, Lesay A, Arcaro A, Lopes R, Ng BL, Warne PH, McNeish IA, Tetley TD, Lemoine NR, Mehmet H, Seckl MJ and Downward J. (2003). *Mol. Cell. Biol.*, **23,** 7600–7610.

Reed JC. (2003). *Cancer Cell*, **3,** 17–22.

Riedl SJ, Renatus M, Schwarzenbacher R, Zhou Q, Sun C, Fesik SW, Liddington RC and Salvesen GS. (2001). *Cell*, **104,** 791–800.

Salvesen GS and Duckett CS. (2002). *Nat. Rev. Mol. Cell. Biol.*, **3,** 401–410.

Scott FL, Denault JB, Riedl SJ, Shin H, Renatus M and Salvesen GS. (2005). *EMBO J.*, **24,** 645–655.

Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J, Lee RA, Robbins PD, Fernandes-Alnemri T, Shi Y and Alnemri ES. (2001). *Nature*, **410,** 112–116.

Sun C, Cai M, Gunasekera AH, Meadows RP, Wang H, Chen J, Zhang H, Wu W, Xu N, Ng SC and Fesik SW. (1999). *Nature*, **401,** 818–822.

Sun Y, Bian J, Wang Y and Jacobs C. (1997). *Oncogene*, **14,** 385–393.

Thompson CB. (1995). *Science*, **267,** 1456–1462.

Wolf BB, Schuler M, Echeverri F and Green DR. (1999). *J. Biol. Chem.*, **274,** 30651–30656.

Wu G, Chai J, Suber TL, Wu JW, Du C, Wang X and Shi Y. (2000). *Nature*, **408,** 1008–1012.

Wyllie AH. (1980). *Nature*, **284,** 555–556.

Wyllie AH, Kerr JF and Currie AR. (1980). *Int. Rev. Cytol.*, **68,** 251–306.

Yang L, Cao Z, Yan H and Wood WC. (2003a). *Cancer Res.*, **63,** 6815–6824.

Yang L, Mashima T, Sato S, Mochizuki M, Sakamoto H, Yamori T, Oh-Hara T and Tsuruo T. (2003b). *Cancer Res.*, **63,** 831–837.

Yang YL and Li XM. (2000). *Cell Res.*, **10,** 169–177.